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# Source:sink imbalance detected with leaf- and canopy-level spectroscopy in a field-grown crop

Angela C. Burnett  | Shawn P. Serbin  | Alistair Rogers 

Environmental and Climate Sciences  
Department, Brookhaven National Laboratory,  
Upton, New York

## Correspondence

Angela C. Burnett, Department of Plant  
Sciences, University of Cambridge, Cambridge,  
UK.

Email: acb219@cam.ac.uk

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## Abstract

The finely tuned balance between sources and sinks determines plant resource partitioning and regulates growth and development. Understanding and measuring metabolic indicators of source or sink limitation forms a vital part of global efforts to increase crop yield for future food security. We measured metabolic profiles of *Cucurbita pepo* (zucchini) grown in the field under carbon sink limitation and control conditions. We demonstrate that these profiles can be measured non-destructively using hyperspectral reflectance at both leaf and canopy scales. Total non-structural carbohydrates (TNC) increased 82% in sink-limited plants; leaf mass per unit area (LMA) increased 38% and free amino acids increased 22%. Partial least-squares regression (PLSR) models link these measured functional traits with reflectance data, enabling high-throughput estimation of traits comprising the sink limitation response. Leaf- and canopy-scale models for TNC had  $R^2$  values of 0.93 and 0.64 and %RMSE of 13 and 38%, respectively. For LMA,  $R^2$  values were 0.91 and 0.60 and %RMSE 7 and 14%; for free amino acids,  $R^2$  was 0.53 and 0.21 with %RMSE 20 and 26%. Remote sensing can enable accurate, rapid detection of sink limitation in the field at the leaf and canopy scale, greatly expanding our ability to understand and measure metabolic responses to stress.

## KEYWORDS

carbohydrates, *Cucurbita pepo*, development, food security, growth, metabolic profiles, remote sensing

## 1 | INTRODUCTION

Plant breeders are faced with a significant challenge: the need to develop high-yielding crop varieties, which are resilient to future climate change (Ainsworth, Rogers et al., 2008; Ort et al., 2015; Simkin, López-Calcano, & Raines, 2019). Recent work in model crops has demonstrated that improvements in yield can be realized by engineering more efficient photosynthesis (Degen, Worrall, & Carmo-Silva, 2020; Kromdijk et al., 2016; Li et al., 2020; López-Calcano et al., 2020; South, Cavanagh, Liu, & Ort, 2019). However, whilst increased  $\text{CO}_2$  assimilation (carbon source activity) can be shown to enhance yield, the response is often not matched by an equivalent

increase in plant growth and yield (carbon sink activity). Elevated  $\text{CO}_2$  research, including free-air concentration enrichment (FACE) experiments, has shown that relatively large stimulations in photosynthesis do not always translate to commensurate increases in growth and yield providing evidence for a sink limitation bottleneck (Ainsworth, Leakey, Ort & Long 2008; Leakey et al., 2009; Long, Ainsworth, Leakey, Nösberger, & Ort, 2006; Sanz-Sáez et al., 2010). Thus, carbon sink limitation can reduce the potential for enhanced yield resulting from genetically engineered improvements to source activity and the anticipated yield benefit of rising atmospheric  $[\text{CO}_2]$ . To fully capitalize on genetically enhanced and  $\text{CO}_2$ -stimulated photosynthesis, carbon sink limitation must be minimized. Indeed, an integrated

understanding of carbon sources and sinks is increasingly recognized as a vital component of enhancing global food production (Ferne et al., 2020; Smith, Rao, & Merchant, 2018; White, Rogers, Rees, & Osborne, 2016). If breeders are to achieve the goal of minimizing sink limitation (Ferne et al., 2020), it is essential to understand how carbon sink limitation may be measured in a high-throughput manner, in order to facilitate rapid screening for sink limitation in breeding programs (Reynolds & Langridge, 2016).

We address this need by defining the metabolic signature of carbon sink limitation in field-grown *Cucurbita pepo* and then developing a high-throughput system for measuring this sink limitation non-destructively using “hyperspectral”, or high spectral resolution, reflectance data. Unlike several other limitations on growth that are of interest to breeders, such as disease resistance or drought resilience in response to pathogen or water stress, there is often no visual phenotype associated with sink limitation. This means that effective measurement of sink stress relies upon destructive harvesting, making continual monitoring difficult to achieve. Furthermore, carrying out the biochemical analysis required for detailing the sink stress profile is time consuming and expensive. For these reasons, a non-destructive, high-throughput, spectroscopic approach to monitoring sink stress is a desirable tool for crop breeders.

For the first time, we examine the source:sink balance of field-grown plants using hyperspectral data. The metabolic profile of carbon sink limitation has been extensively characterized (Bénard et al., 2015; Burnett, Rogers, Rees, & Osborne, 2016; Stitt & Krapp, 1999). Chief among the key traits of a carbon sink-limited plant is an increase in leaf carbohydrate content, which has been phenomenologically and mechanistically linked to reduced sink strength (Ainsworth & Bush, 2011; Farrar, 1996; Pollock & Cairns, 1991; Rogers & Ainsworth, 2006). It has also been well established that reflectance data can be used to estimate a suite of leaf traits using a range of approaches, including empirical partial least-squares regression (PLSR) modelling to build relationships between spectral data and measured traits. This spectra-trait PLSR approach provides a rapid, high-throughput means for the estimation of biochemical traits of interest (Cotrozzi & Couture, 2020; Ely, Burnett, Lieberman-Cribbin, Serbin, & Rogers, 2019; Meacham-Hensold et al., 2019; Serbin, Singh, McNeil, Kingdon, & Townsend, 2014; Silva-Perez et al., 2018; Yendrek et al., 2017), including those associated with source-sink balance and carbon and nitrogen status. Here, we build on these advances in two key ways: we evaluate the spectroscopic approach for identifying sink limitation in the production environment and we scale up from leaf- to canopy-level acquisition of hyperspectral data using a boom-mounted spectrometer – a key step in moving to truly high-throughput monitoring using remote sensing tools. We evaluated the spectra-trait modelling approach in field-grown *C. pepo* with a sink removal treatment, to determine if the relationships between reflectance and leaf traits could be used for predicting key indicators of source:sink imbalance in the field. We also investigated the capability of linear discriminant analysis (LDA) and partial least squares discriminant analysis (PLS-DA) for identification of metabolic stress using measured leaf traits and using raw hyperspectral data, respectively. These discriminant analyses were used to build class-prediction models which demonstrated the use of trait and

spectral data to distinguish between plants in the control or sink removal treatment. Importantly, we demonstrate with each of our approaches that sink limitation may be successfully detected at both the leaf and canopy scale.

We tested the following hypotheses: (a) There will be significant metabolic and structural differences between leaves of sink-limited and control field-grown *C. pepo* plants. (b) These metabolic and structural differences can be detected in the field using hyperspectral reflectance data acquired at the leaf scale. (c) These metabolic and structural differences can also be detected remotely using hyperspectral reflectance data collected at the canopy scale.

## 2 | MATERIALS AND METHODS

### 2.1 | Plant material and experimental treatments

There are diverse ways to experimentally manipulate the carbon source:sink balance, including: defoliation, debudding or sink removal; manipulation of temperature, light, nitrogen or CO<sub>2</sub> levels; and transgenic modifications (Ainsworth, Rogers, Nelson, & Long, 2004; White et al., 2016). Direct manipulations of the carbon sink in field experiments are comparatively rare. Here, we reduced the carbon sink of field-grown *C. pepo* by removing developing fruits, throughout the duration of the experiment. The continual removal of fruits is a standard agricultural practice for harvesting zucchini, although we removed fruits early in their development.

*C. pepo* was selected for this experimental work because it rapidly forms a full canopy, making this species an ideal target for our proof-of-concept study of canopy reflectance. *C. pepo* is a suitable model crop for sink manipulation experiments because its fruits are highly visible due to large colourful flowers, there are relatively few fruits per plant, and the fruits are easily removed. Furthermore, the local environment is suitable for growing *C. pepo*, which is commonly cultivated as a commercial crop on Long Island.

Seeds of *C. pepo* L. var. Dunja were obtained from the Long Island Cauliflower Association (Riverhead, New York, USA) and grown in a research field at Brookhaven National Laboratory, Upton, New York, USA in 2019 (latitude 40.864466, longitude 72.875158, 18 m elevation). Following initial pH testing, the field was prepared with lime prior to sowing to ensure an appropriate soil pH for *C. pepo*. Seeds were sown on DOY 158 at a density to achieve full canopy coverage in eighteen 10 m × 10 m plots, each surrounded by a border of *C. pepo*, of the same width as 1.5 times the height of mature *C. pepo* (estimated from previous experiments) to give a total sown area of 12 m × 12 m. The large plot size was selected to facilitate canopy- and UAS-level data collection. In addition to the sink manipulation experiment described here, the field was also used for a drought experiment. Six plots underwent sink manipulation and six plots underwent drought treatment; six plots served as controls. For the sink manipulation treatment, developing fruits were removed from each plant twice per week beginning on DOY 196 by using a short-bladed serrated harvesting knife to slice the midpoint of the short

fleshy stem at the base of the fruit. The use of a complete, regularly maintained sink removal treatment gave rise to large variation in metabolite contents in order to fill the “trait space” (the numerical range of data points), facilitating the development of robust PLSR models. For the drought treatment, following germination and plant establishment, irrigation was withheld from drought plots resulting in soil dry-down during periods of no precipitation. Irrigation was maintained at standard local agricultural levels in the sink manipulation and control plots. The drought treatment began on DOY 186 and lasted until the end of the experiment. Data from drought plots were not used in the main study presented here but were included in PLSR models to increase model performance by further increasing the range of trait values, thus giving more robust data prediction capabilities. Data were collected from each of the three plot types on every measurement date. Meteorological data are reported in Figure S1.

## 2.2 | Experimental schedule

Leaf and canopy spectral data were collected twice per week for the duration of the experiment (from the initiation of the sink removal treatment until senescence of the control plants). All spectral data collection and leaf harvesting was always performed within 3 hr of solar noon (i.e., between 10:00 and 16:00 EDT) because canopy spectral data collection requires the sun to be high in the sky to provide even illumination of the leaves. Leaf harvests for obtaining biochemical traits were alternately paired with either leaf spectral data collection or canopy spectral data collection on any given day, in order to facilitate the development of PLSR models at both the leaf and canopy scales. Conducting full diurnal time courses of leaf metabolite measurements was beyond the scope of this work and not possible given the constraints of sampling canopy spectra around solar noon. However, the strong experimental treatment used to build predictive models should enable the prediction of metabolite contents at different times of day via leaf level spectral data, and this will be an important future application of the work presented here.

## 2.3 | Leaf spectral data

For leaf spectral data collection, three sets of random coordinates were selected on each measurement day and applied to each plot. The newest fully expanded leaf at each coordinate was selected for measurement. First, leaf temperature was measured using a handheld infrared radiometer (Apogee Instruments, Logan, Utah, USA). Immediately afterwards, spectral data were collected using a PSR+ full-range (continuous 350–2,500 nm) spectroradiometer (Spectral Evolution, Lawrence, Massachusetts, USA), connected to a leaf clip assembly with an internal, calibrated light source (SVC, Poughkeepsie, New York, USA). The spectroradiometer was calibrated using a LabSphere Spectralon® reflectance standard disc (LabSphere, Inc., North Sutton, New Hampshire, USA). For each leaf, three spectral measurements were taken across the adaxial surface and then averaged to give a single spectrum.

## 2.4 | Canopy spectral data

For canopy spectral data collection, three evenly spaced locations were measured within the central region of each plot. The spectroradiometer was fitted with a 14° lens (Spectral Evolution, Lawrence, Massachusetts, USA) and positioned 2 m above the canopy on a truck-mounted boom (Figure 4b). The spectroradiometer was calibrated using a LabSphere Spectralon® reflectance standard plate (LabSphere, Inc., North Sutton, New Hampshire, USA). Leaf temperature in the area viewed by the spectroradiometer was first measured using the infrared radiometer, positioned above the canopy. For each location within the plot, three to five spectral measurements were performed (immediately following temperature measurement) and then averaged to give a single spectrum. When it was not possible to measure canopy spectra on all experimental plots due to logistical or weather constraints, the omitted plots were noted and prioritized for measurement on the next measurement date, ensuring an overall even coverage of canopy spectral data for all experimental plots.

## 2.5 | Physiological data

Measurements of  $\Phi_{PSII}$  and relative chlorophyll content were performed using the PhotosynQ MultispeQ V 2.0 (Kuhlgert et al., 2016) on the newest fully expanded leaf immediately following leaf spectral data collection. The MultispeQ measurements were made on light-adapted leaves, at ambient conditions matching the incident photosynthetically active radiation (PAR) and the temperature at the leaf surface, using the “Photosynthesis RIDES” protocol available online at [photosynq.org](http://photosynq.org).

In addition to the regular measurements of  $\Phi_{PSII}$  performed on all plots throughout the experiment, the response of photosynthesis to intercellular  $CO_2$  concentration ( $A/C_i$  curves) was performed on a subset of plots on four dates. However, this data collection was constrained by instrument availability. These data, presented in Figure S2, were collected in the field between the hours of 06:00 and 14:00, using a LI-6800 Portable Photosynthesis System (LI-COR Biosciences, Lincoln, Nebraska, USA). A diurnal measurement of photosynthesis was performed prior to measurement of  $A/C_i$  curves to ensure that measurements were completed before the onset of the afternoon suppression of photosynthesis occurring naturally in plants in all treatments. Light response curves were performed to determine the saturating irradiance to be used in  $A/C_i$  curves: 2000  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ .  $A/C_i$  curves were performed on the newest fully expanded and physiologically mature leaf. Leaves were acclimated in the leaf cuvette until steady-state  $A$  and  $g_s$  were reached (20–45 min). Each  $A/C_i$  curve began at 400  $\mu\text{mol mol}^{-1} CO_2$ , and the  $CO_2$  concentration was decreased, then increased in a stepwise manner as described previously (Rogers, Serbin, Ely, Sloan, & Wullschlegel, 2017).

Steady-state values of  $C_i/C_a$  and  $A_{sat}$  at 400  $\mu\text{mol mol}^{-1} CO_2$  were obtained from the first point of the  $A/C_i$  curve.  $V_{c,max}$  was estimated from  $A/C_i$  response curves and should therefore be considered as apparent  $V_{c,max}$  since mesophyll conductance was not measured,

meaning that values are based on intercellular rather than chloroplastic [ $\text{CO}_2$ ]. Estimation of  $V_{c,\max}$  was made using the kinetic parameters and their temperature dependence as presented previously (Bernacchi et al., 2013; Bernacchi, Singsaas, Pimentel, Portis, & Long, 2001) following the method described in detail by Rogers et al. (2017). The average root-mean-squared error (RMSE) associated with fitting  $V_{c,\max}$  was 1.26 (<1% of estimated  $V_{c,\max}$ )  $\pm$  0.88 (SD). Values of  $V_{c,\max}$  were normalized to 25°C using an Arrhenius function (Bernacchi et al., 2013).

## 2.6 | Sample collection

Leaves were either harvested immediately following leaf spectral data collection for each plant or following canopy spectral data collection for each area of the plot. For harvests paired with leaf spectra, the leaf that had been used for spectral data collection was harvested. For harvests paired with canopy spectra, the newest fully expanded leaf from the centre of the area viewed by the spectroradiometer was harvested. Each leaf was divided into two equal halves along the midrib. Discs from one half were punched evenly across the leaf surface, placed into an aluminium foil packet and immediately flash frozen in liquid nitrogen, at the field site. The second half of the leaf was kept intact and sealed in a plastic bag containing a damp paper towel, to prevent desiccation during the sampling of the remaining leaves. These intact leaf halves were placed into a cooler at the field site to prevent sample deterioration. After harvesting was complete, all samples were returned to the laboratory. Frozen samples were stored at  $-70^\circ\text{C}$  for subsequent biochemical analysis. The intact half of each leaf was punched into discs of known area distributed evenly across the leaf surface, weighed and transferred to a drying oven for the subsequent determination of leaf mass per unit leaf area (LMA) and leaf water content (LWC).

## 2.7 | Leaf trait analysis

Leaf mass per unit leaf area was obtained from the measured area and dry mass of oven-dried leaves. LWC was obtained from leaf fresh mass at time of harvest and leaf dry mass after oven drying, according to the following formula:

$$\text{LWC (\%)} = \frac{(\text{leaf fresh mass} - \text{leaf dry mass})}{\text{leaf fresh mass}} \times 100.$$

Analysis of leaf carbon- and nitrogen-containing metabolites (glucose, fructose, sucrose, starch, amino acids and protein) was performed as described previously (Burnett et al., 2016). In brief, sequential ethanol extractions were used to extract metabolites from frozen tissue. For sugars (glucose, fructose and sucrose), a continuous enzymatic substrate assay was performed in the presence of ATP and NADP; the NADPH signal associated with each sugar was measured at 340 nm (ELx808 Plate Reader, BioTek, Winooski, VT, USA). Amino

acids were quantified using fluorescamine in the presence of sodium borate buffer, with fluorescence measured at 360 nm excitation, 460 nm emission and 40 nm bandwidth (Synergy HT Plate Reader, BioTek, Winooski, VT, USA) after 5 min dark incubation. Protein was quantified from the pellets resulting from the ethanol extraction using a commercially available kit (Pierce BCA protein assay kit, ThermoFisher Scientific, Rockford, IL, USA) following solubilization in 0.1 M sodium hydroxide. For starch, pellet samples were first neutralized with hydrochloric acid following the protein assay. An overnight enzymatic digest was performed, and the resultant sugars were quantified as described above. For each assay, a standard curve was included on every plate to ensure accurate metabolite quantification. For a more detailed description of these methods, refer to Burnett et al. (2016). Biochemical traits were expressed on a per unit area basis, derived from the relationship between fresh mass and leaf area that was obtained from the oven-dried samples.

Values of carbohydrate-corrected LMA (ccLMA) were obtained from the total non-structural carbohydrate data (TNC; the sum of glucose, fructose, sucrose and starch) as follows. TNC, expressed as mmol glucose equivalents  $\text{m}^{-2}$ , was first multiplied by the millimolar mass of glucose to give TNC,  $\text{g m}^{-2}$ . This value was then multiplied by the area of each sample to give TNC,  $\text{g sample}^{-1}$ . Finally, ccLMA ( $\text{g m}^{-2}$ ) was obtained using the following equation:

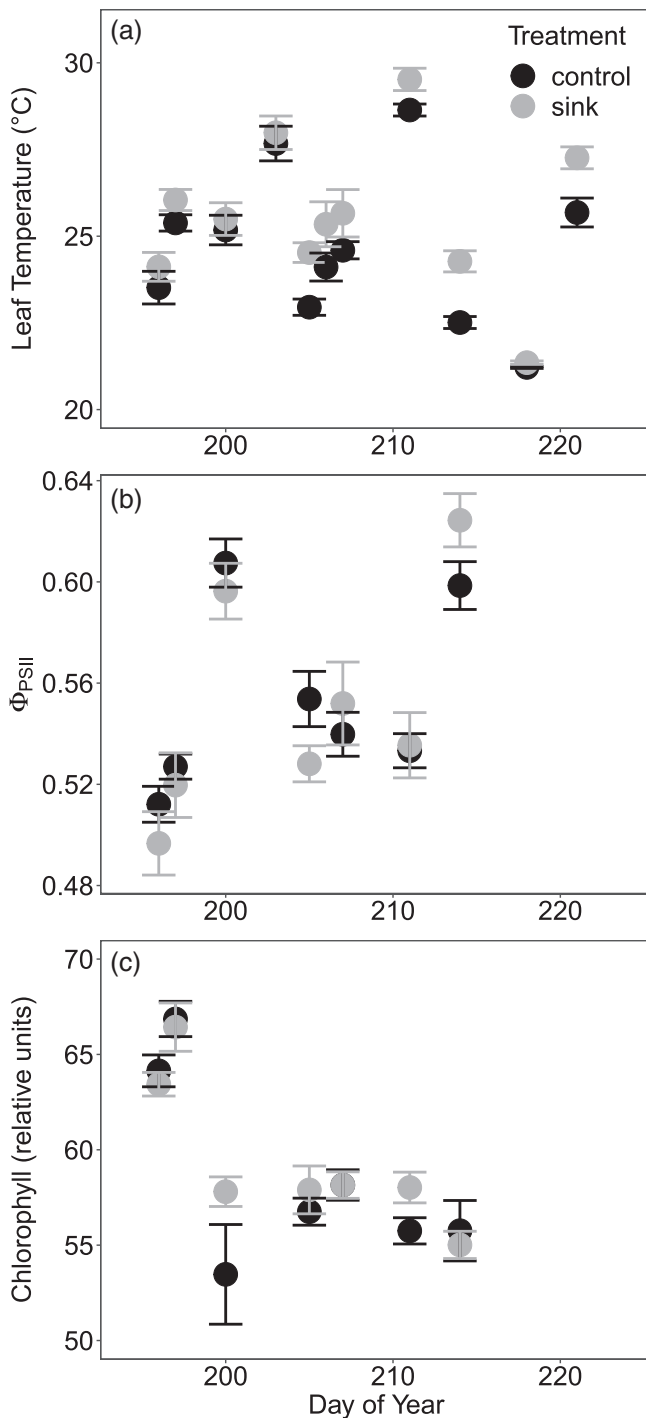
$$\text{ccLMA} = \frac{(\text{sample dry mass} - \text{sample TNC mass})}{\text{sample area}}.$$

## 2.8 | UAS flight data

An unoccupied aerial system (UAS) flight was performed on DOY 226, the last day of the measurement period, using an “Osprey” system as described previously (D. Yang et al., 2020). Since the flight was carried out at the end of the measurement period, only the RGB data were analysed for this study; the full data are available online as detailed at the end of the manuscript. Green Chromatic Coordinate (GCC; Richardson, 2019) was obtained for each experimental plot using RGB camera data following the methods of D. Yang et al. (2020). This metric enables standardization of RGB data between different cameras, facilitating comparison with future work.

## 2.9 | Data analysis

All data analysis was performed in the R open source software environment (R Core Team, 2019). For analysis of leaf traits, depicted in Figures 1 and 2 and Table 1, each trait was analysed using repeated-measures ANOVA. Analysis was performed at the plot level ( $n = 6$  plots for each treatment). For each measurement date, the within-plot values obtained from three leaves per plot were first averaged to give one value for each trait per plot and per measurement date. Next, if required, data were log- or square-root-transformed prior to analysis to satisfy requirements for normally distributed data. ANOVA was



**FIGURE 1** Leaf temperature was higher in sink-limited plants, but photosynthesis-related traits did not change. (a) leaf temperature; (b) efficiency of photosystem II ( $\Phi_{PSII}$ ); (c) relative chlorophyll content. For leaf temperature (a)  $n = 6$  plots each reporting an average value of 3–6 reps per plot measured at leaf and/or canopy scales on any given date; the final two time points show the average of five sink-limited plots and four control plots. For MultispeQ measurements (b,c)  $n = 6$  plots with an average of three reps taken for the plot-level value. Means  $\pm$  SE of plot-level data are shown

used to test for effects of and interactions between treatment categories (control and sink-limited) and time into the sink manipulation experiment (DOY), with repeated measurements at the plot level. The

significance level was set to  $p < .05$ , with individual significance levels reported in Table 1. A post-hoc Tukey test was performed to determine the dates upon which differences between treatments were significant.

PLSR was used to predict leaf traits from spectral data using the “pls” package (Mevik & Wehrens, 2007) in R. PLSR models included drought plants, control plants and sink-limited plants to increase the predictive power by extending the range of trait values and the number of samples. Measured starch and TNC data were square-root-transformed prior to modelling; untransformed data are always presented in the manuscript. A small set of samples was removed from the dataset prior to fitting, due to outlier residual errors, for each trait.

Leaf-level PLSR models were built using all spectral wavelengths between 500 and 2,400 nm with the exception of TNC and free amino acids when the range 1,100–2,400 nm was used to improve accuracy of model prediction. Canopy-level PLSR models were built using the spectral wavelengths 500–1,800 nm and 1950–2,400 nm in order to eliminate the 1800–1950 nm region containing atmospheric water interference. For canopy-level PLSR models for TNC and starch, the starting wavelength was 1,100 nm rather than 500 nm to improve the model fit. Spectral data did not undergo any transformation prior to model building.

For all PLSR models, observational data points were subset according to treatment then randomly assigned to datasets for calibration (80% of the data) and validation (20% of the data) (Table 2). Component selection and model calibration were carried out as described previously (Ely et al., 2019; Serbin et al., 2014). The  $R^2$  and RMSE of prediction of the validation data set were used to assess each model, and the variable importance of projection (VIP) was used for qualitative evaluation of model predictor variables as described previously (Wold, Sjöström, & Eriksson, 2001).

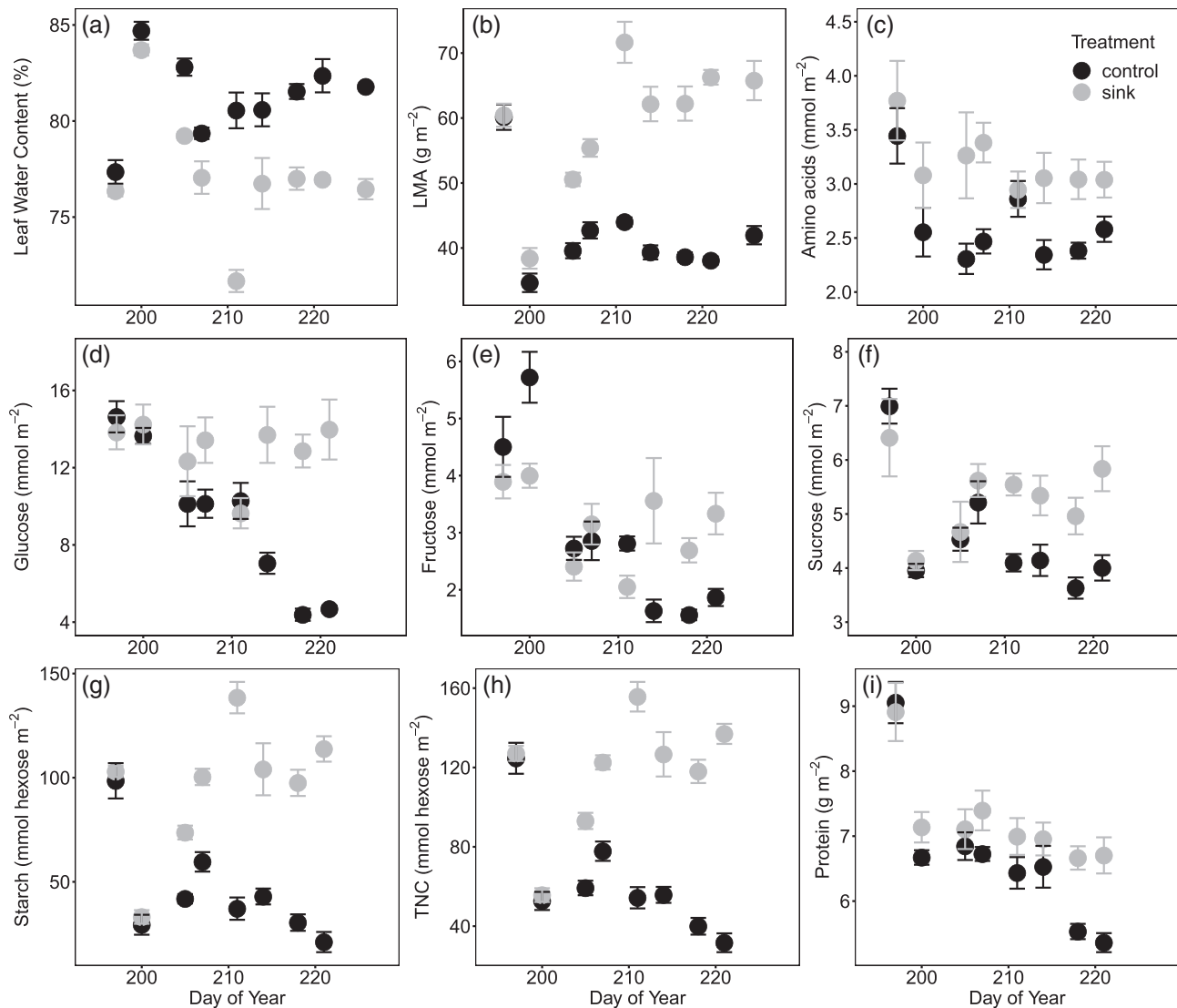
PLS-DA was performed in R using the “caret” package (Kuhn, 2008), in accordance with methods developed previously (Cotrozzi & Couture, 2020; Ely et al., 2019; Gold et al., 2020; Serbin et al., 2014). PLS-DA models used 75% of the data for model training and 25% for model testing, with 10-fold cross-validated resampling repeated five times, and receiver-operator curves (ROC) optimization of the number of components. Models ran until convergence was reached (up to 100 iterations). The LDA model was built using 75% of the data for model training and 25% for model testing; the LDA model was trained with leave-one-out calibration and ROC-optimization of the number of components. The average results from 10 model iterations are reported.

GCC was analysed using a  $t$ -test of plot-level mean GCC values for sink-limited and control plots (Figure S3).

### 3 | RESULTS

#### 3.1 | Photosynthesis is maintained in sink-limited plants

The sink removal treatment began on day of year (DOY) 196, and plants were measured from the onset of treatment until DOY 226 when control plants were senescing. Physiological, metabolic and



**FIGURE 2** Leaf metabolic and structural traits in sink-limited (grey points) and control (black points) plants. (a) leaf water content (LWC); (b) leaf mass per unit area (LMA); (c) free amino acids; (d) glucose; (e) fructose; (f) sucrose; (g) starch; (h) TNC and (i) protein. TNC is the sum of glucose, fructose, sucrose and starch. For leaf metabolic traits (c–i),  $n = 6$  plots with the exception of measurements made 9, 15 and 25 days into treatment when five sink-limited plots and four control plots were measured. The data for each plot represent an average value from three harvested leaves. For leaf structural traits (a,b) sampling was identical to leaf metabolic traits with the exception of the final time point on which samples were taken for LWC and LMA only, with  $n = 2$  sink-limited and  $n = 2$  control plots. Means  $\pm$  SE of plot-level data are shown

structural traits were measured (Figures 1 and 2). Averaged over the experiment, leaf temperature was 3% higher in sink limited plants; leaf temperature was 6% higher in sink limited plants at the final time point. Temperature differences were significant across the experiment taken as a whole; when individual measurement dates were analysed, significant differences in temperature occurred on DOY 205, 206, 214, 221 (Figure 1a; Table 1). Photosystem II operating efficiency (Figure 1b; Table 1) was not affected by the sink manipulation. Leaf chlorophyll content was not affected by the sink manipulation except for DOY 200 when it was significantly higher in sink manipulated plants (Figure 1c; Table 1). The lack of overall photosynthetic response displayed in data collected throughout the experiment (Figure 1b) was supported by a small dataset of  $A/C_i$  curves which

showed no clear trend in  $A_{sat}$  or  $V_{c,max}$  although  $g_s$  was lower in sink-limited plants ( $F_{1,8} = 7.2$ ,  $p < .05$ ; Figure S2).

### 3.2 | Sink limitation affects leaf metabolism and structure

Leaf structure was markedly affected by the sink manipulation treatment. There was a 5% decrease in LWC in the sink manipulation treatment, and the magnitude of this effect increased with time, with a highly significant time  $\times$  treatment interaction (Figure 2a; Table 1). We observed a 38% increase in LMA (mean for all time points) overall, displaying the highest increase of 57% at the final time point, and a



**TABLE 1** Leaf traits shown in Figures 1 and 2 analysed with repeated-measures ANOVA

Trait	Effect	F-value	p-value
Leaf temperature	Treatment	$F_{(1,8)} = 20.1$	$p < .01$
	Time	$F_{(10,94)} = 59.6$	$p < .001$
	Time $\times$ Treatment	$F_{(10,94)} = 1.1$	ns
$\Phi_{PSII}$	Treatment	$F_{(1,10)} = 0.3$	ns
	Time	$F_{(6,60)} = 29.2$	$p < .001$
	Time $\times$ Treatment	$F_{(6,60)} = 1.4$	ns
Chlorophyll	Treatment	$F_{(1,10)} = 1.1$	ns
	Time	$F_{(6,60)} = 32.5$	$p < .001$
	Time $\times$ Treatment	$F_{(6,60)} = 1.5$	ns
Leaf water content	Treatment	$F_{(1,6)} = 104.5$	$p < .001$
	Time	$F_{(8,63)} = 27.4$	$p < .001$
	Time $\times$ Treatment	$F_{(8,63)} = 6.0$	$p < .001$
LMA	Treatment	$F_{(1,6)} = 190.7$	$p < .001$
	Time	$F_{(8,63)} = 53.3$	$p < .001$
	Time $\times$ Treatment	$F_{(8,63)} = 21.8$	$p < .001$
Free amino acids	Treatment	$F_{(1,8)} = 14.8$	$P < 0.01$
	Time	$F_{(7,61)} = 4.9$	$p < 0.001$
	Time $\times$ Treatment	$F_{(7,61)} = 1.4$	ns
Glucose	Treatment	$F_{(1,8)} = 13.0$	$p < 0.01$
	Time	$F_{(7,61)} = 13.6$	$p < 0.001$
	Time $\times$ Treatment	$F_{(7,61)} = 12.3$	$p < 0.001$
Fructose	Treatment	$F_{(1,8)} = 1.4$	ns
	Time	$F_{(7,61)} = 20.1$	$p < 0.001$
	Time $\times$ Treatment	$F_{(7,61)} = 9.1$	$p < 0.001$
Sucrose	Treatment	$F_{(1,8)} = 12.4$	$P < 0.01$
	Time	$F_{(7,61)} = 14.3$	$p < 0.001$
	Time $\times$ Treatment	$F_{(7,61)} = 3.8$	$p < 0.01$
Starch	Treatment	$F_{(1,8)} = 134.9$	$p < .001$
	Time	$F_{(7,61)} = 35.5$	$p < .001$
	Time $\times$ Treatment	$F_{(7,61)} = 20.4$	$p < .001$
TNC	Treatment	$F_{(1,8)} = 199.8$	$p < .001$
	Time	$F_{(7,61)} = 37.4$	$p < .001$
	Time $\times$ Treatment	$F_{(7,61)} = 28.8$	$p < .001$
Protein	Treatment	$F_{(1,8)} = 10.9$	$p < .05$
	Time	$F_{(7,61)} = 29.8$	$p < .001$
	Time $\times$ Treatment	$F_{(7,61)} = 2.3$	$p < .05$

Note: Effects of treatment (control and sink manipulation), time (DOY) and the interactive effect are shown.

**TABLE 2** Numbers of datapoints in calibration (cal.) and validation (val.) datasets and number of model components (nComps) for partial least square regression (PLSR) models presented in Figure 5

Trait	Leaf-scale PLSR			Canopy-scale PLSR		
	Cal.	Val.	nComps	Cal.	Val.	nComps
LWC	192	49	7	71	20	9
LMA	190	48	9	71	19	11
Free amino acids	187	48	10	70	19	10
Glucose	191	49	11	70	18	6
Fructose	189	48	11	68	18	5
Sucrose	188	49	9	70	18	9
Starch	180	47	10	67	19	6
TNC	178	47	9	67	19	6
Protein	189	50	8	71	19	8

highly significant time  $\times$  treatment interaction (Figure 2b; Table 1). The difference between control and sink-limited plants was significant on DOY 200 for LMA and for both LMA and LWC on all measurement dates from DOY 205 onwards (Table 2). About half of the overall increase in the raw LMA data presented here was attributable to an increased TNC content in sink-limited plants; carbohydrate-corrected values of LMA (ccLMA) still showed an overall 20% increase in sink-limited plants (see Data S1).

All leaf metabolites were analysed on an area basis. Free amino acid content increased by 22% in sink-limited plants and decreased over time in both control and sink-limited plants with no significant interaction (Figure 2c; Table 1). However, for individual dates, the difference was significant on DOY 200, 205, 207, 214 and 218.

Sink-limited plants displayed a strong and highly significant increase in TNC, which became more marked over time (Figure 2h; Table 1). The increase in TNC was attributable in part to an increased sugar content (Figure 2d–f; Table 1) but was dominated by a marked increase in starch (Figure 2g; Table 1). Glucose was significantly higher in sink-limited plants on DOY 207, 214, 218, 221; fructose was significantly lower in sink-limited plants on DOY 200, then significantly higher on DOY 214, 218, 221; sucrose was significantly higher in sink-limited plants on DOY 211, 214, 218 and 221. Both starch and TNC were significantly higher in sink-limited plants on all measurement dates from DOY 205 onwards. Overall, TNC increased by 82% in sink-limited plants. There was a highly significant time  $\times$  treatment interaction for each carbohydrate measured; the difference between control and sink-limited plants increased as time progressed (Figure 2d–h; Table 1).

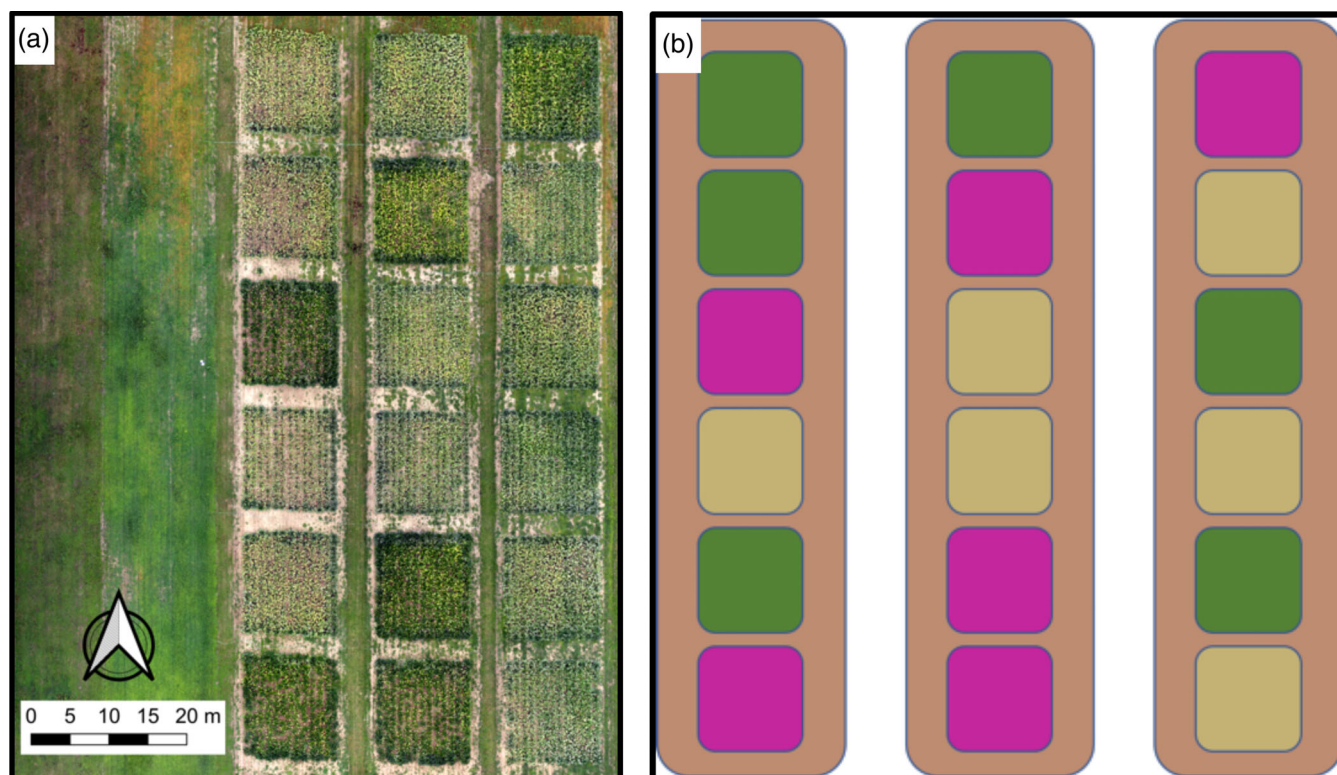
Leaf protein content increased 8% overall in sink-limited plants, and the magnitude of this difference was greatest at the final time point when protein was 25% higher than in the control plants; there was a significant time  $\times$  treatment interaction and the difference between treatments was significant on DOY 207, 218 and 221 (Figure 2i; Table 1).

### 3.3 | Plot-level greenness is maintained in sink-limited plants

Sink-limited plots stayed green for longer than control plots at the end of the experiment, due to delayed senescence (Figure 3). Using UAS imagery, we observed that the Green Chromatic Coordinate (GCC) was significantly higher in sink-limited than control plants ( $t = 5.8$ ,  $p < .001$ ,  $df = 10$ ; Figure S3). This response was also visually evident in the standard red-green-blue (RGB) image where sink-limited plots were visually greener compared to the control plots (Figure 3a).

### 3.4 | Partial least-squares regression successfully predicts metabolic and structural traits from reflectance at leaf and canopy scales

Reflectance data were collected at both leaf and canopy scales using a leaf clip (Figure 4a) and truck-mounted boom (Figure 4b). At both scales, PLSR successfully estimated leaf metabolite contents and structural traits associated with sink limitation (Figure 5). In general,



**FIGURE 3** An aerial image of the field (a) shows a prolonged green phenotype in plants which underwent the sink removal treatment. Plot positions are indicated as follows: green = control, beige = drought, pink = sink removal (b). Drought plants were not used in the main study but were included in PLSR model building to increase model performance (see Methods)





**FIGURE 4** Photographs show spectral data collection at leaf (a) and canopy (b) levels. The spectroradiometer is referenced using a white Spectralon® disc for leaf-level measurements and a white Spectralon® plate for canopy-level measurements, shown in (b)

models performed better at the leaf level, with a higher  $R^2$  when compared to the canopy-level model for the same trait, and a lower RMSE for six of nine leaf-level models when compared to canopy-level models (Figure 5; data shown are for independent model validation in each case).  $R^2$  values for leaf-level models ranged from 0.53 to 0.93 demonstrating strong predictive capabilities (Figure 5). Canopy-level models also showed acceptable predictive capabilities for many traits. Five of the nine models had  $R^2$  values greater than 0.5 (Figure 5) with the highest  $R^2$  of 0.78 for LWC. The %RMSE (RMSE expressed as a percentage of the mean of the observed values for a trait) ranged from 2 to 37% for leaf models, and from 2 to 38% for canopy models. For leaf models, %RMSE was <20% for all traits except sugars and was 2% for LWC, 7% for LMA, 20% for free amino acids, 11% for protein and 13% for TNC. For canopy models, %RMSE was 2% for LWC, 14% for LMA, 26% for free amino acids, 10% for protein and 38% for TNC.

### 3.5 | Sink stress detection may be achieved using measured traits or hyperspectral reflectance

LDA was used to determine whether or not plants were exposed to sink stress, based on metabolic and structural traits (glucose, fructose, sucrose, starch, protein, free amino acids, LMA and LWC). When LDA was performed iteratively, including cumulative data for each successive date and the preceding dates, class detection accuracy improved over time as the treatment effect became stronger. The maximum overall accuracy was 86% when all time points were included (Figure 6), and the area under the receiver-operator curve (AUC-ROC) was 0.93. PLS-DA using raw spectral data also showed a good capability for distinguishing between sink-limited and control plants, with detection success of 78% at the leaf level (AUC-ROC = 0.86) and 89% for canopy level spectra (AUC-ROC = 0.96), including all measured time points (Figure 6). The greater success of detection with canopy spectra is likely due to the fact that compared to leaf-scale

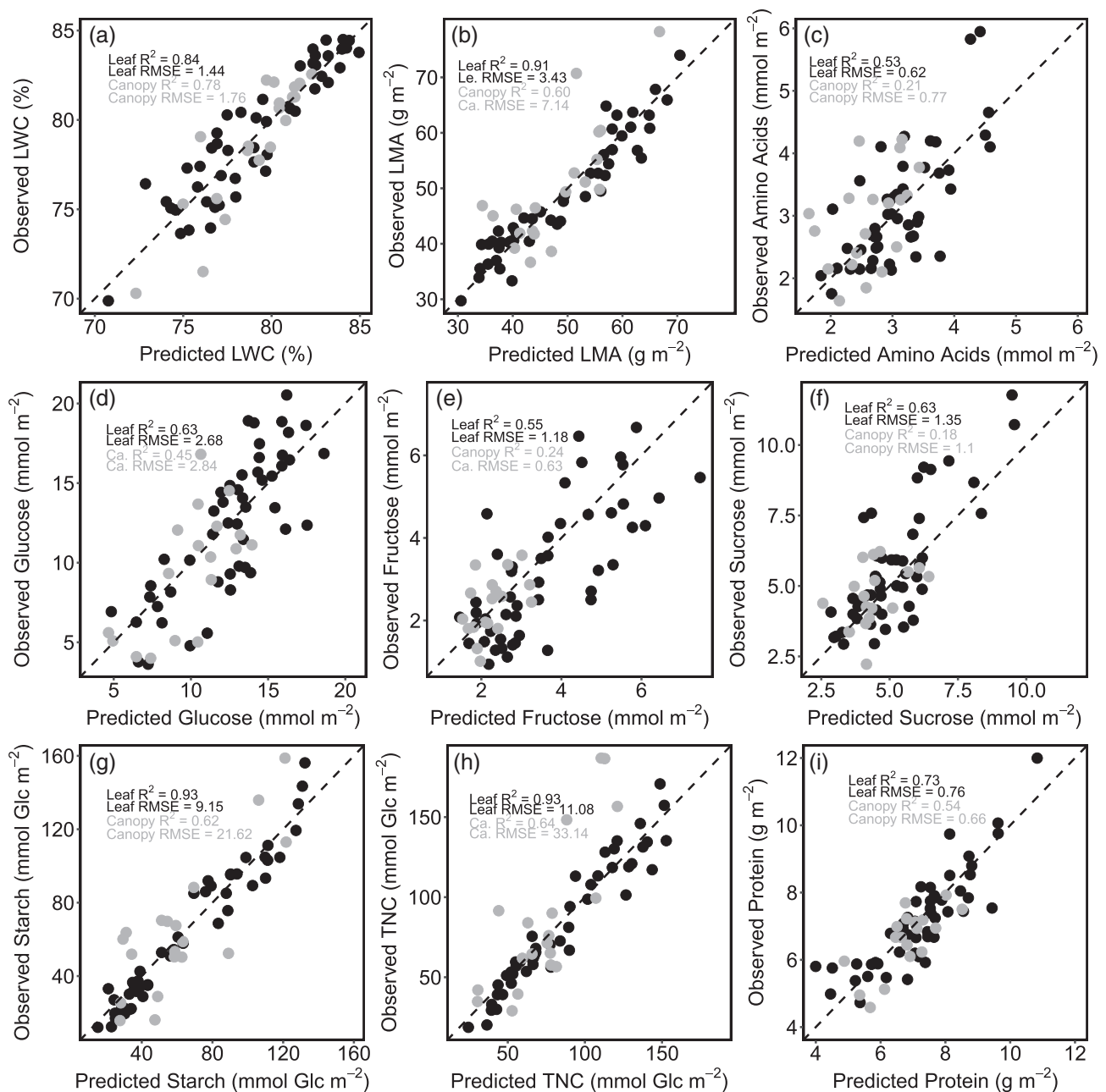
data collection, canopy spectral data collection began slightly later into the experiment. This would enhance the overall treatment effect observed in canopy data, since the metabolic differences between treatments generally increased over time as the sink stress became more pronounced. After we omitted the leaf measurements that did not overlap with those from the canopy collections in the PLS-DA (i.e., leaf and canopy measurement periods were aligned, with the earliest part of the experiment omitted) the detection accuracy at the leaf scale was 93% (AUC-ROC = 0.99). The equivalent measurement for LDA using measured leaf traits yielded a prediction accuracy of 94% (Figure 6). Measured traits and hyperspectral reflectance are both successful at distinguishing between sink-limited and control plants (Figure 6).

## 4 | DISCUSSION

We conducted a sink manipulation experiment in field-grown *C. pepo* and demonstrated that we could detect the marked and significant effect of sink limitation (hypothesis a) on leaf metabolic and structural traits using spectroscopy. Our key finding was that this approach can be scaled effectively from the leaf level to the canopy scale (hypotheses b and c). Collectively, our results demonstrate the robustness of the spectroscopy approach, the potential to detect sink limitation non-destructively and remotely and to do that in a real-world agricultural setting, emphasizing the value of the approach for breeders and producers.

### 4.1 | The metabolic signature of sink stress

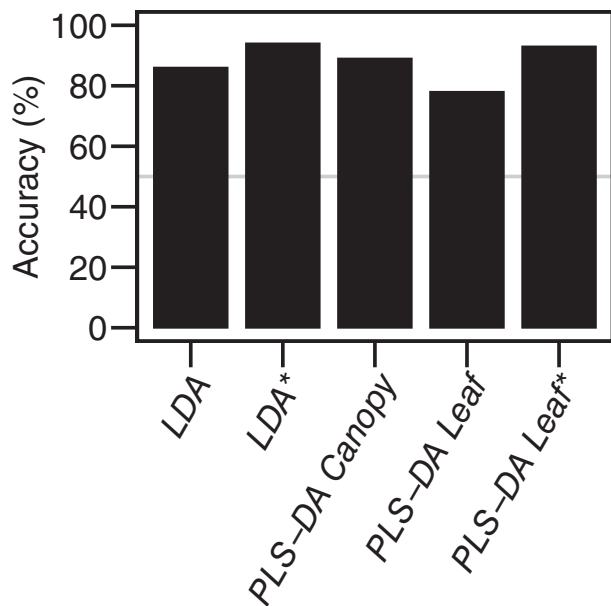
Sink strength is the product of sink size multiplied by sink activity (Geiger & Shieh, 1993; White et al., 2016). Removing developing fruits dramatically decreases carbon sink strength within the plant, by removing a critical carbon sink. However, fruit removal also increases



**FIGURE 5** Partial least squares regression (PLSR) models demonstrate that spectral data may be used to predict leaf structural (a,b) and metabolic (c-i) traits at both leaf and canopy scales. Each plot shows the validation results for leaf-level (black points) and canopy-level (grey points) models. Plots show the relationship between observed traits from traditional measurements and predicted traits derived from spectral data. The dashed line shows the 1:1 relationship, and  $R^2$  and RMSE values are provided for each model (black and grey text are used for leaf and canopy models, respectively). For leaf PLSR models, calibration datasets included between 178 and 192 datapoints, and validation datasets included between 47 and 50 datapoints (Table 2). For canopy PLSR models, there were between 67 and 71 calibration datapoints and between 18 and 20 validation datapoints (Table 2)

the sink activity by stimulating the development of new fruits, which have a strong carbon requirement, thereby increasing sink strength within the plant. In the manipulation performed here, the net effect on sink strength was an overall decrease, since the increase in sink activity was outweighed by the larger decrease in sink size, and this is confirmed by the trends in carbohydrate levels observed in sink-limited plants (Figure 2d-h).

Like most biotic and abiotic plant stresses, sink limitation has a metabolic signature. Here, sink limitation led to significant increases in the content of non-structural carbohydrates (Figure 2d-h) and free amino acids (Figure 2c) in addition to leaf structural changes (Figure 2a,b). Increased levels of non-structural carbohydrates – both each carbohydrate metabolite individually and the total pool (TNC) – are consistent with the literature on carbon sink limitation; our sink



**FIGURE 6** Linear discriminant analysis (LDA) using measured leaf traits and partial least squares discriminant analysis (PLS-DA) using raw spectral data measured at leaf and canopy scales successfully predict whether or not plants were sink limited. The grey line represents the 50% (equal to chance) detection rate. AUC-ROC values are reported in the text. Asterisks indicate when a subset of data was used, with aligned leaf and canopy measurement periods, to facilitate comparison with canopy-level data, for which collection began later than leaf-level data

removal treatment for manipulation of the source:sink balance, therefore, elicited the expected response. Sink-limited plants had increased levels of leaf carbohydrates likely due to decreased export from the leaf caused by reduced sink demand (Ainsworth & Bush, 2011; Burnett et al., 2016; Stitt & Krapp, 1999).

The observed leaf structural changes (LWC and LMA; Figure 2a,b; Table 1) are consistent with the development of smaller, longer-lasting leaves; furthermore, these leaf characteristics are themselves consistent with the delayed leaf senescence and maintained leaf protein content observed in sink-limited plants (Figures 2i and 3). Delayed leaf senescence has been observed in multiple FACE experiments in which elevated  $[\text{CO}_2]$  increased the carbon source:sink balance non-destructively, further indicating that our findings are commensurate with a source:sink imbalance (Kontunen-Soppela et al., 2010; McGrath, Karnosky, & Ainsworth, 2010; Tallis et al., 2010).

## 4.2 | Detection of metabolic traits at leaf and canopy scales using hyperspectral reflectance

Since the sink limitation treatment was not accompanied by a change in photosynthesis (Figures 1 and 2; Table 1; Figure S2), screening for sink limitation in zucchini plants requires insight into the metabolic response. Biochemical measurements are not only time-consuming and costly to perform but are also destructive, meaning that a leaf-

level study cannot track an individual leaf over its lifespan. In addition, the delay in obtaining results from destructive analysis is substantial, preventing rapid feedback to breeders or farmers. Therefore, the use of high-throughput, non-destructive hyperspectral data, which can readily be analysed to understand the metabolic status of a plant (at the leaf level) or field plot (at the canopy level), enables a great step forward in the efficiency and capability of sink stress monitoring. Our study provides the first field-level example of non-destructive monitoring of sink stress via metabolite prediction.

Whilst leaf-level PLSR models generally had a higher capability for predicting traits from spectral data than their canopy-level counterparts (Figure 5), models at both scales were effective at predicting a suite of leaf traits. Importantly, predictions of starch, which forms the vast majority of TNC, and leaf structural traits (LWC and LMA) were successful at both leaf and canopy levels with  $R^2 > 0.60$  in each case (Figure 5a,b,g,h). Since TNC was the major metabolic indicator of sink limitation in this study, this indicates effective prediction of sink limitation at the canopy scale. To our knowledge, this is the first time that canopy-level predictive models have been used to examine the traits underpinning crop sink limitation.

## 4.3 | Perspectives on scaling up trait detection

Scaling detection of traits from the leaf level to the canopy level is a critical step to enable high-throughput measurement of plant traits (Asner & Martin, 2008; Herrmann et al., 2018; Kokaly, Asner, Ollinger, Martin, & Wessman, 2009; Virlet, Sabermanesh, Sadeghi-Tehran, & Hawkesford, 2017). To measure traits at the canopy level, the time of day must be carefully considered, given the reliance on natural illumination of the leaves by solar irradiation, rather than artificial illumination from the light sources typically used in a leaf clip. Leaf orientation and canopy structure also become relevant for canopy-scale measurements of reflectance (Ollinger, 2011); gaps between plants must be avoided in order to obtain a reliable spectral measurement of the core vegetation component. Finally, atmospheric water vapour can interfere with the signal in the main water absorption regions (Gao, Heidebrecht, & Goetz, 1993) and must be removed from the spectral data prior to analysis. Here, we successfully demonstrated the use of canopy-level spectral data for detecting sink limitation, representing a major advance in the phenotyping of sink limitation – a recognized critical target for crop breeding (Dusenge, Duarte, & Way, 2019; Fernie et al., 2020).

Scaling the hyperspectral monitoring of sink stress to the UAS level using UAS-mounted hyperspectral sensors (Shiklomanov et al., 2019; G. Yang et al., 2017; D. Yang et al., 2020) is the next step for increasing throughput and the ability to scale the technique, as has been shown for phenotyping of wheat height in response to a nitrogen treatment (Holman et al., 2016) and canopy characteristics of avocado trees (Tu, Johansen, Phinn, & Robson, 2019). However, it must be noted that scaling up to the UAS level is not without its technical, economic and legislative challenges (Coops, Goodbody, & Cao, 2019; Hunt & Daughtry, 2018). In terms of technical limitations, UAS-

mounted spectral cameras often have a narrower range of wavebands and a lower waveband resolution than hyperspectral sensors used on the ground, for example 10 nm resolution in the study by Basso, Fiorentino, Cammarano, and Schulthess (2016), reducing measurement precision. In the present study, we used the Green Chromatic Coordinate (GCC), which is a simple metric derived from RGB camera data, to demonstrate that sink-limited plots were greener than control plots at the end of the experimental period, measured at the UAS level. This finding is likely related to underlying physiological and biochemical traits, since GCC is linked to pigments and plant health as well as leaf area index (Liu et al., 2015; Liu, An, Lu, Hu, & Tang, 2018; Reid, Chapman, Prescott, & Nijland, 2016). Simple metrics such as GCC provide a less expensive approach to airborne crop monitoring, and UAS measurements frequently rely on spectral indices rather than taking the full-spectrum trait prediction approach demonstrated using our leaf- and canopy-level data. However, for detecting small changes and understanding the underlying metabolic differences, hyperspectral data provide far more detailed information than spectral indices. Hyperspectral data – in contrast to multi-spectral data – are especially suited to measurements of nutrient status as well as other stresses such as pathogens when using UAS systems (Maes & Steppe, 2019), and it will be important to use high spectral resolution when scaling up our detailed approach for stress detection from the leaf- and canopy-level to the UAS level.

The detection of metabolic and structural signatures using leaf reflectance facilitates faster screening for crop breeding as well as the development of precision agriculture techniques. In the plant breeding context, understanding sink limitation – whether at the leaf, canopy or field scale – enables the development of crops better able to translate additional photosynthate resulting from improved carbon assimilation (Degen et al., 2020; Kromdijk et al., 2016; Li et al., 2020; López-Calcano et al., 2020; South et al., 2019) or future elevated CO<sub>2</sub> (Ainsworth, Rogers, & Leakey, 2008; Leakey et al., 2009) into enhanced yield. In the precision agriculture context, monitoring sink limitation in major crops may be used to inform the timing of fertiliser application to improve the balance between carbon and nitrogen resources in the plant (Basso et al., 2016; Maes & Steppe, 2019; Maresma, Ariza, Martínez, Lloveras, & Martínez-Casasnovas, 2016). Both carbon and nitrogen can place limits on crop growth, development and yield (Burnett et al., 2016; Burnett, Rogers, Rees, & Osborne, 2018; White et al., 2016), and both source and sink limitations must be addressed for successful breeding of our future crops (Ferne et al., 2020; White et al., 2016).

In summary, source:sink balance underpins plant growth and survival and is a key factor affecting crop yield. In order to realize crop yield increases, an integrated understanding of carbon and nitrogen sources and sinks is essential. Remote sensing provides a unique opportunity for detailed, high-throughput phenotyping of plant physiological and metabolic traits, enabling us to understand limitations on yield caused by sink limitation. Here, we have demonstrated the use of leaf reflectance data to examine vital plant processes in the production environment, measuring source:sink balance remotely in field-grown plants for the first time, enabling rapid and non-invasive measurements of sink limitation.

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## CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

## DATA AVAILABILITY STATEMENT

The spectra and trait data presented in this manuscript are available on-line [http://ecosis.org] from the Ecological Spectral Information System (EcoSIS) at <https://doi.org/10.21232/RLmYbmE3>.

The UAS dataset used in this manuscript is available on-line at <https://doi.org/10.17605/OSF.IO/SN6EM>.

Additionally, the spectra and trait data presented here, along with the carbohydrate-corrected LMA dataset, metadata, and a ReadMe text file, are available as Data S1 accompanying this manuscript.

## ORCID

Angela C. Burnett  <https://orcid.org/0000-0002-2678-9842>

Shawn P. Serbin  <https://orcid.org/0000-0003-4136-8971>

Alistair Rogers  <https://orcid.org/0000-0001-9262-7430>

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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